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Agrobacterium*-mediated transformation of *Campanula glomerata

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Abstract A transformation system for *Campanula glomerata* ‘Acaulis’ based on the co-cultivation of leaf explants with *Agrobacterium tumefaciens* LBA4404 or EHA105 was developed. *A. tumefaciens* was eliminated when the explants were cultured on medium containing 400 mg/l vancomycin and 100 mg/l cefotaxime. Transgenic plants containing the *uidA* gene that codes for β -glucuronidase (*gus*) were obtained following co-cultivation with either strain of *A. tumefaciens*, LBA4404 or EHA105, both of which harbored the binary vector pGUSINT, coding for the *uidA* and neomycin phosphotransferase II (*nptII*) genes. While the transformation frequency (2–3%) was similar for both strains, *A. tumefaciens* LBA4404 was effectively eliminated from *Campanula* at a lower concentration of antibiotic as compared to EHA105. The concentration of individual antibiotics required to eliminate EHA105 resulted in a decreased rate (55–67%) of regeneration. The highest percentage of explants that regenerated plants (79%) and the highest regeneration rate was achieved with 100 mg/l cefotaxime combined with 400 mg/l vancomycin. Plants were also transformed with the isopentenyl transferase (*ipt*) gene using LBA4404 containing the 35S-*ipt* vector construct (pBC34).

Keywords *Agrobacterium tumefaciens* EHA105 and LBA4404 · Bellflower · *ipt* gene · *nptII* gene · *uidA* gene

Abbreviations BA: Benzyladenine · CMV 35S: Cauliflower mosaic virus 35S · 2,4-D: 2,4-Dichlorophenoxyacetic acid · *gus*: β -Glucuronidase · *ipt*: Isopentenyl transferase · NAA: α -Naphthaleneacetic acid · *nptII*: Neomycin phosphotransferase II

Introduction

The genus *Campanula*, commonly known as ‘bellflower’, is diverse with approximately 300 species recorded for the temperate region of the northern hemisphere (Crook 1951). Most *Campanula* species are used as both bedding and pot plants, and some species are used as cut flowers. *C. glomerata* ‘Acaulis’ is a dwarf form, which makes it useful as either a bedding plant for the border or a pot plant (Lewis and Lynch 1989). Since 1993 bedding plants have represented the largest growing segment of the floral industry, and in 1999 the wholesale value of bedding plants was 1.95 billion dollars, up 4% from 1998 (Floriculture Crops, National Agricultural Statistics Service 1999). There is always a demand by the public for more variety among the floral crops, and genetic engineering offers the possibility to develop plants with new traits.

The isopentenyl transferase (*ipt*) gene is one of the tumor-inducing genes of *Agrobacterium tumefaciens* and codes for the enzyme isopentenyl transferase, a key enzyme in cytokinin biosynthesis (Akiyoshi et al. 1984; Barry et al. 1984). A high level of endogenous cytokinin has been shown to produce transgenic plants with a lack of apical dominance, increased shoot formation (Keel et al. 1987; Smigocki 1991), and delayed senescence (Gan and Amasino 1995). We therefore expected ‘Acaulis’ plants transformed with the *ipt* gene to show an increased number of shoots and flower stems, which would be a desirable characteristic for marketing as a pot plant.

There are a few reports available on the micropropagation of *Campanula isophylla* (Brandt 1992, 1994), but

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no papers have been published on a shoot regeneration system and its application to genetic transformation. A system for regenerating shoots from leaf explants of *C. glomerata* 'Acaulis' grown in vitro was developed, and it was found that both temperature and light intensity were critical factors that affected propagation in vitro and subsequent regeneration from the leaf explants (Joung et al. submitted).

Several species of *Campanula* (*medium*, *glomerata*, *lactiflora*, and *rotundifolia*) have been transformed with *Agrobacterium rhizogenes* for the establishment of hairy root cultures with increased levels of secondary metabolites (Tada et al. 1996; Tanaka et al. 1999; Ishimaru et al. 2001). Direct regeneration of shoots occurred following culture of the roots on half-strength MS (Murashige and Skoog 1962) medium lacking hormones; however there was no information on the frequency of shoot regeneration, and Southern hybridization of DNA blots was not presented to verify that the shoots were transformed and free of *Agrobacterium* (Ishimaru et al. 2001). Here we report a method for *A. tumefaciens*-mediated transformation of *C. glomerata* 'Acaulis' plants using LBA4404. We could demonstrate that the transgenic plants contained either the pGUSINT binary vector with its *nptII* gene and the *uidA* gene coding for gus expression or the pBC34 vector coding for the 35S-*ipt* gene.

Materials and methods

In vitro culture of stock plants

Campanula glomerata L. 'Acaulis' plants were cultured on MS medium (M-9274, Sigma Chemical, St. Louis, Mo.) supplemented with 3% sucrose and the following (in mg/l): thiamine, 0.1; nicotinic acid, 0.5; pyridoxine, 0.5; myo-inositol, 100; NAA, 0.01; BA, 1.0, and solidified with 0.8% phytagar, pH 6.5. The MS medium and all supplements were autoclaved at 120°C and 20 psi for 20 min, except for zeatin and all of the antibiotics, which were filter-sterilized and added to the sterilized cool medium. Four plantlets were cultured in a magenta jar (Sigma) at 14°C under continuous light provided by cool-white fluorescent lights at an intensity of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were subcultured every 3 months and used as the source of leaf explants. Each in vitro-grown leaf was cut at the proximal and distal end and served as a single leaf explant – 0.4–0.5 cm wide \times 0.3–0.4 cm long – for subsequent experiments.

Antibiotic analysis

Carbenicillin (Sigma C1388), cefotaxime (Sigma C7912), and vancomycin (Sigma V2002) were used to eliminate *Agrobacterium*, and kanamycin (Sigma K4000) was used for the selection of transgenic plants. To determine the optimum antibiotic concentration that eliminated *Agrobacterium*, we tested the antibiotics either alone or in combination by streaking *Agrobacterium* on solidified YEB medium (5.0 g/l Difco Bacto beef extract, 5.0 g/l peptone, 1.0 g/l yeast extract, 5.0 g/l sucrose) supplemented with antibiotic(s) and 1.5% Difco Bacto agar, pH 7.2.

The optimum antibiotic concentration for maximum shoot regeneration was determined by placing *Campanula* leaf explants on MS medium supplemented with 4 mg/l zeatin, 0.2 mg/l NAA, and antibiotics, pH 6.5. Three explants per petri dish (60 \times 20 mm) were placed adaxially on the MS medium, and the petri dishes were placed in a growth chamber maintained at 18°C under con-

tinuous (24-h cycle) light of low intensity (10 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The explants were subcultured to fresh medium every 3 weeks. Regeneration data were collected after 100 days in culture.

Bacterial strains

For the transformation of *Campanula* with the *uidA* gene, disarmed *Agrobacterium tumefaciens*, EHA105 (pEHA105) (Hood et al. 1993) and LBA4404 (pAL4404) (Hoekema et al. 1983), containing the binary vector p35SGUSINT (Vancanneyt et al. 1990) consisting of the *uidA* gene containing an intron under the CaMV 35S promoter and the *nptII* gene under the nos promoter were used. For *ipt*-gene transformation, LBA4404 containing the pBC34 vector (Smigocki and Owens 1988) with the *ipt* gene controlled by the CaMV 35S promoter and the *nptII* gene under the nos promoter was used. Strain EHA105 was maintained at 28°C on YEB medium containing 10 mg/l kanamycin, 1.5% Difco Bacto agar, pH 7.2. Strain LBA4404 was maintained on YEB medium supplemented with 250 mg/l streptomycin.

Regeneration, transformation, and selection

Prior to co-cultivation, agrobacteria were grown overnight in YEB liquid medium containing 50 μM acetosyringone. Leaf explants were soaked in the *Agrobacterium* suspension for 15 min, then transferred to sterile blotting paper to remove the excess bacterial suspension. Explants were transferred abaxial side down onto co-cultivation medium (MS medium with 2 mg/l 2,4-D, pH 6.5) and incubated in the dark at 26°C for 2–3 days. After co-cultivation, the explants were transferred to regeneration medium (MS medium supplemented with 4 mg/l zeatin, 0.2 mg/l NAA) containing 50 mg/l kanamycin for selection and both 400 mg/l vancomycin and 100 mg/l cefotaxime for the elimination of *Agrobacterium*, pH 6.5. Explants were subcultured to fresh medium every 3 weeks until shoots regenerated. Regenerated shoots were excised from the callus and transferred to MS medium supplemented with 50 mg/l kanamycin, 400 mg/l vancomycin, and 100 mg/l cefotaxime.

DNA analysis

DNA for the polymerase chain reactions (PCR) was extracted from putative transformants according to the method of Wang et al. (1993). The *uidA* gene was amplified using the primer sequences 5'-gtt ggg cag gcc agc gta tcg tg-3' and 5'-taa cct tca ccc ggt tgc cag agg-3' and thermal cycler conditions of: 1 cycle of 95°C for 5 min and 35 cycles of 94°C for 30 s, 62°C for 30 min, 72°C for 1 min, followed by 3 min at 72°C. The *ipt* gene was amplified using the primer sequences 5'-cat cta att ttc ggt cca act tgc a-3' and 5'-cga tat cca tcg atc tct t-3' and thermal cycler conditions of: 1 cycle at 95°C for 5 min and 35 cycles of 94°C for 30 s, 54°C for 30 min, 72°C for 1 min, followed by 3 min at 72°C.

DNA for Southern hybridizations was isolated from leaves of in vitro-grown plants using the DNeasy Plant Mini Kit (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. DNA was digested with *EcoRI*, electrophoresed on an 0.8% agarose gel in 0.5 \times TBE (45 mM Tris-borate, 45 mM boric acid, 3 mM EDTA, pH 8) buffer, and then transferred to a Nytran membrane (Schleicher and Schuell, Keene, N.Y.) by capillary blotting (Maniatis et al. 1982). For hybridization with the *uidA* gene, a 511-bp PCR-amplified *uidA* gene product labeled using digoxigenin (DIG)-dUTP (PCR DIG Probe Synthesis kit, Boehringer Mannheim, Indianapolis, Ind.) was used as the probe. For hybridization with the *ipt* gene, a 515-bp DIG-labeled PCR-amplified *ipt* fragment was used. Hybridization and detection of the DIG-labeled nucleic acid was performed using the DIG Easy Hyb and DIG Nucleic Acid Detection kits (Boehringer Mannheim). DNA cross-linked Nytran membranes were incubated at 42°C for 16 h with the probe for hybridization and then washed two times at

26°C, 5 min each wash, in 2× SSC, 0.1% SDS, followed by two washes, 15 min each wash, at 65°C in 0.1× SSC, 0.1% SDS.

Gus analysis

Regenerated shoots were assayed for expression of the *uidA* gene following the histochemical staining procedure described by Jefferson et al. (1987) with some modifications. Shoots were incubated 16 h at 37°C in staining solution [100 mM sodium phosphate, pH 7, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM Na₂EDTA, 0.5% (v/v) Triton X-100] with 0.5 mg/l 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). After 16 h of staining, the shoots were cleared and fixed in a solution of 75% (v/v) ethanol:1% (v/v) acetic acid.

Zeatin analysis

Plants were subcultured on MS medium without plant growth regulators for 1 month prior to analysis of endogenous zeatin. The leaves were harvested, frozen in liquid nitrogen, and stored at -70°C until analyzed. Zeatin (zeatins, zeatin ribosides, and derivatives of zeatin) were extracted according to the instructions provided with the Zeatin kit (De Dansk Sukkerfabrikker, Copenhagen). Extracted zeatins were quantified by an ELISA technique using the Phytodetek t-ZR kit (Agdia, PDK 09348/0096, Elkhart, Ind.). The analysis was replicated three times for each plant line, and zeatin levels were averaged.

Results and discussion

Effect of antibiotic(s) on regeneration

The regeneration rate of the leaf explants decreased when cultured on increasing concentrations (100–400 mg/l) of

either cefotaxime, carbenicillin, or vancomycin (Table 1). For individual antibiotics, either 400 mg/l cefotaxime, 800 mg/l vancomycin, or 500 mg/l carbenicillin was required to kill *Agrobacterium* strain EHA105. In comparison, strain LBA4404 was killed at lower concentrations of all three antibiotics – 200 mg/l cefotaxime, 400 mg/l vancomycin, or 250 mg/l carbenicillin. The regeneration frequency was reduced to 55–67% of the explants when individual antibiotics that killed EHA105 were used (Table 1); therefore, several combinations of two antibiotics were tested to determine the optimum combination/concentration for eliminating *Agrobacterium* without severely inhibiting regeneration. The highest percentage of explants that regenerated plants (79%) and the highest regeneration rate was achieved with 100 mg/l cefotaxime combined with 400 mg/l vancomycin. The use of LBA4404 was found to be preferable to EHA105 for *Campanula* transformation since a lower concentration of antibiotic successfully eliminated LBA4404 (Table 1) and growth of EHA105 was observed in EHA105-infected leaf explants cultured for over 1 month on MS medium supplemented with 100 mg/l cefotaxime and 400 mg/l vancomycin. The supervirulent strains EHA101 and EHA105 (Hood et al. 1986, 1993) are effective in transformation (Lulsdorf et al. 1991; DeBont et al. 1994), but they have not been used for some crops because bacterial growth cannot be controlled effectively (Maheswaran et al. 1992) as was found to be the case with *Campanula*.

Kanamycin was used for the selection of putative transformants. The minimum concentration of kanamycin

Table 1 Effect of antibiotics on the regeneration of *Campanula* from leaf explants and growth of two *Agrobacterium* strains, EHA105 and LBA4404. The regeneration data was collected after

100 days of culture on MS medium supplemented with 4 mg/l zeatin and 0.2 mg/l NAA

Antibiotics (mg/l)		Regeneration ^a	Regeneration	Bacterial growth ^c	
		Rating	Percentage of total explants ^b	EHA105	LBA4404
Control		4.2±0.5	100g	+	+
Cefotaxime (Cef)	100	3.7±0.4	88e,f,g	±	±
	200	2.9±0.4	69b,c	+	–
	400	2.3±0.3	55a	–	–
Vancomycin (Van)	200	4.1±0.7	98f,g	±	±
	400	3.5±0.4	83d,e	+	–
	800	2.8±0.5	67a,b,c	–	–
Carbenicillin (Car)	250	3.6±0.3	86d,e,f	+	–
	500	2.7±0.3	65a,b,c	–	–
	750	2.4±0.3	57a,b	–	–
Cef+Van ^d	200+200	3.0±0.8	69b,c	–	–
	100+400	3.3±0.4	79c,d	–	–
	200+400	2.5±0.5	60a,b	–	–
Car+Van ^d	200+400	3.1±0.5	74c,d	–	–

^a Regeneration rating: 0, explant dead; 1, only callus formed; 2, one shoot regenerated; 3, two shoots regenerated; 4, three shoots regenerated; 5, four shoots regenerated; 6, more than four shoots regenerated. Each value represents the mean ± SE of three replications, each with 15 leaf explants per treatment

^b Means with different letters are significantly different at $P \leq 0.01$ according to the Tukey test

^c *Agrobacterium* was streaked on solidified YEB medium containing antibiotic and cultured at 26°C for 5 days. +, Growth; ±, bacteria grown on only part of streak line; –, no bacterial growth

^d Cef+Van, Car+Van: combined treatment of either cefotaxime or carbenicillin with vancomycin

Table 2 Frequency of putatively transformed shoots regenerated from *C. glomerata* 'Acaulis' leaf explants co-cultivated with *Agrobacterium* strains EHA105 or LBA4404 on MS medium con-

taining 50 mg/l kanamycin, 4 mg/l zeatin, 0.2 mg/l NAA, 400 mg/l vancomycin, and 100 mg/l cefotaxime

	<i>Agrobacterium</i> strain		
	EHA105 (pGUSINT)	LBA4404 (pGUSINT)	LBA4404 (pBC34)
Number of explants	102	81	51
Number of surviving calluses ^a	28 (28%) ^d	11 (14%)	31 (61%)
Number of explants forming shoots ^b	12 (12%)	6 (7%)	14 (27%)
Number of Southern-positive shoots ^c showing transgene expression	2 (2%)	2 (3%)	6 (12%)

^a Two months after co-cultivation

^b Three months after co-cultivation

^c Four months after co-cultivation

^d Percentage of total number of explants in parenthesis

cin required to kill non-transformed leaf explants of *Campanula* was 50 mg/l, and death of the explant occurred after 3 months of culture.

Selection and analysis of the gus transgenic plants

Calluses formed from explants 2 months following co-cultivation on MS medium supplemented with 4 mg/l zeatin, 0.2 mg/l NAA, 400 mg/l vancomycin, 100 mg/l cefotaxime, and 50 mg/l kanamycin for selection (Table 2). Twelve shoots (from 12% of the explants) and six shoots (from 7% of the explants) were induced from the calluses 3 months after co-cultivation with EHA105 and LBA4404, respectively. The presence of the *uidA* gene as verified by PCR analysis of putatively transformed plants indicated that seven plants following co-cultivation with EHA105 and three plants following LBA4404 co-cultivation possibly contained the *uidA* gene (Table 2). Only two of the seven PCR-positive plants expressed gus throughout the leaves and petioles following EHA105 infection (Fig. 1). In comparison, two of the three PCR-positive plants expressed gus throughout the leaves and petioles following LBA4404 infection (Fig. 1). There may have been false positives using PCR analysis since PCR will amplify the *uidA* gene in *Agrobacterium* that may have persisted in the regenerated shoots.

Southern analysis of the four transgenic plants that expressed gus verified that each transgenic plant has a single copy of the *uidA* gene (Fig. 2) and that each plant represents an independently transformed line. The plasmid DNA pGUSINT released a 2.8-kb fragment when cut with *Hind*III and *Eco*RI (Fig. 2, lane 1). Each band that hybridized to genomic DNA represents a single copy of the transgene as there is a single *Eco*RI site located at the end of the nos terminator of pGUSINT. The frequency of transformation for stably transformed plant lines that expressed gus in the leaves and petioles was 2% of the total number of explants or 17% of the explants that regenerated shoots using EHA105, and 3% of the total number of explants or 33% of the explants that regenerated shoots using LBA4404.

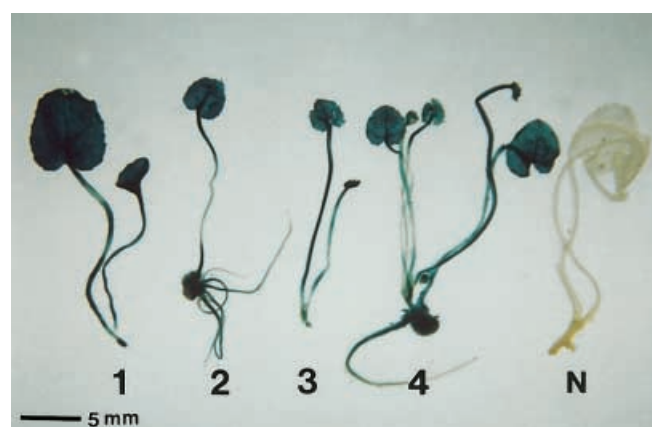


Fig. 1 Gus expression in transgenic *Campanula glomerata* 'Acaulis' leaves. N Non-transgenic plants, 1–4 transgenic plants resulting from co-cultivation with EHA105 (1 and 2) and LBA4404 (3 and 4)

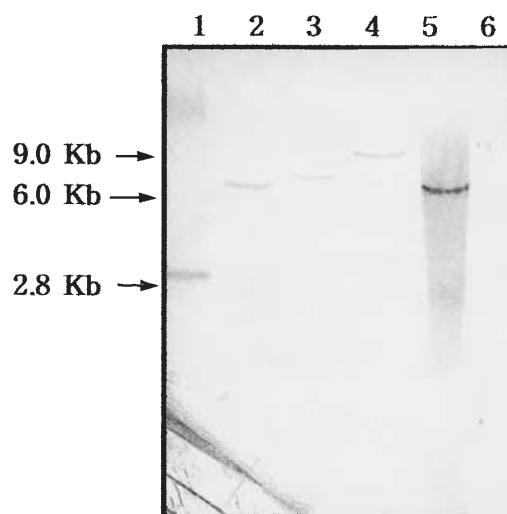


Fig. 2 DNA hybridization with genomic DNA isolated from *C. glomerata* 'Acaulis' plants transformed with the *uidA* gene. Lane 1 pGUSINT was digested with *Eco*RI and *Hind*III and used as a positive control, lanes 2 and 3 gus-positive plants following co-cultivation with EHA105, lanes 4 and 5 gus-positive plants following co-cultivation with LBA4404, lane 6 non-transformed plant. All plant DNA (10 µg) was digested with *Eco*RI and hybridized with the 511-bp *uidA* gene probe

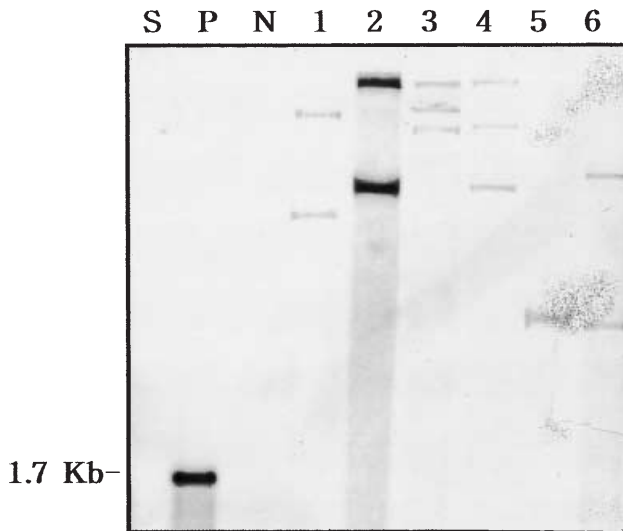


Fig. 3 DNA hybridization with genomic DNA isolated from the *ipt* gene transgenic *C. glomerata* 'Acaulis' plants. *S* Size marker, *P* pBC34 digested with *Eco*RI and *Hind*III and used as the positive control, *N* Non-transformed plant, lanes 1–6 *ipt* gene-transgenic plant lines 34-1, -2, -3, -4, -5, -6, respectively. All plant DNA (10 µg) was digested with *Eco*RI and hybridized with the 515-bp *ipt* gene probe

Selection and analysis of the *ipt* gene transgenic plants

Fourteen shoots (27% of the total number of explants) were induced from the calluses 3 months after co-cultivation. Six shoots were green, and the other eight shoots, which were albino, did not survive on MS medium. Genomic DNA was cut with *Eco*RI only, and because there is only one *Eco*RI site located at the end of the nos terminator following the *ipt* gene, each hybridizing band represents a single copy of the transgene. Southern analysis confirmed that all six green shoots contained the *ipt* gene, and the copy number ranged from one to three (Fig. 3). pBC34 plasmid DNA cut with *Hind*III and *Eco*RI yielded the expected 1.7-kb fragment. The frequency of stable transformation as verified by Southern hybridization was 12% of the total number of explants or 43% of the explants that regenerated shoots (Table 2).

Zeatin concentration in transgenic plants

The endogenous zeatin level in leaves of non-transformed plants averaged 0.08 nmol/g fw (fresh weight). In the six lines of *ipt*-transformed plants, the zeatin levels varied from 3.42 (line 34-4) to 22.60 (line 34-2) nmol/g fw. The zeatin levels in the transgenic plants were 43- to 269-fold greater than those in non-transformed plants (Fig. 4). The leaves of transgenic lines 34-3 and 34-4, which had a 65- and 43-fold increase in the zeatin level, respectively, as compared to non-transformed plants, were green. The other transgenic lines (34-1, 34-2, 34-5, 34-6), which had 90 times more zeatin than the non-transformed plants, produced leaves that

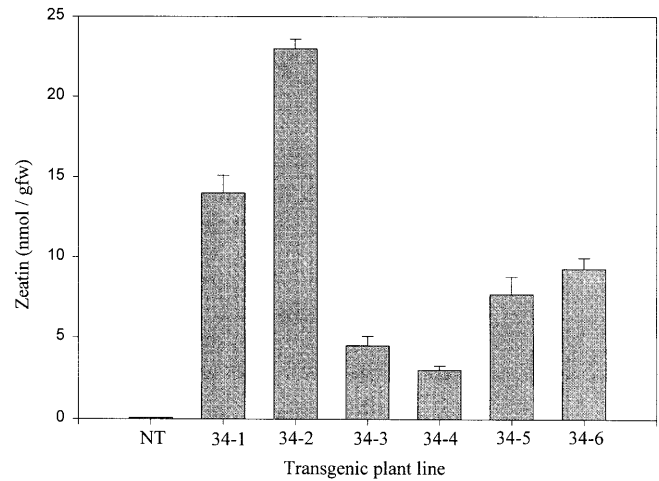


Fig. 4 Zeatin concentration in plants transformed with the *ipt* gene. Each bar represents an average of three samples ± SE. *N* Non-transformed plant, lanes 34-1 through 34-6 *ipt* gene-transformed plants



Fig. 5A–C Phenotypes resulting from *ipt* expression in transgenic *C. glomerata* 'Acaulis'. **A** Non-transformed plant, **B**, **C** transgenic plants 34-4 (**B**) and 34-2 (**C**)

were a lighter shade of green than those of the non-transformed plants when the plants were grown in vitro (Fig. 5). Transgenic tobacco and *Arabidopsis* plants containing *ipt* under the heat shock 70 promoter produced a 52-fold increase in levels of zeatin and a 23-fold increase in zeatin riboside; these levels are significantly lower than the 260-fold increase in zeatin concentration found in transgenic *Campanula* plants (Medford et al. 1989). Albino shoots of tobacco and *Arabidopsis* were not reported by Medford et al. (1989), possibly because the lower levels of zeatin in their transgenic plants did not result in albinism. The non-transformed, regenerated plants of *Campanula* were never observed to be albino, indicating that albinism was not caused by the regeneration conditions used. Possibly the antibiotics used during regeneration caused albinism of the *Campanula* plants.

Transgenic *Nicotiana* plants containing the *ipt* gene under the CaMV 35S promoter showed enhanced shoot formation, lack of apical dominance, and no root forma-

tion (Smigocki and Owens 1988), and these same traits were observed in the transgenic *Campanula* plants containing the *ipt* gene. Roots were not formed when six transgenic lines of *Campanula* containing the *ipt* gene were grown on MS medium without hormones, and 0.5 mg/l NAA was required for root induction. Line 34-4, which contained the lowest concentration of zeatin, has formed roots following culture on NAA, and several plants of line 34-4 have survived transfer to the greenhouse. Transgenic lines 34-1, -2, -5, and -6, with 90 times as much zeatin as non-transformed plants, were phenotypically abnormal, and although their leaves were lighter green than non-transformed plants when grown in vitro (Fig. 5), the leaves became darker green, curved, shinier, and more hardened than those of normal shoots when the transgenic plants were grown in the greenhouse. The growth of the transgenic plants containing the *ipt* gene was relatively slow compared to that of the non-transformed, regenerated plants. Eleven non-transformed, regenerated plants readily developed roots, have been transplanted to the greenhouse, and it is anticipated that they will require 2–3 years of growth in the greenhouse before flowering. This is similar to the time required for *Campanula* plants of a similar size, but derived from seed, to flower. It is anticipated that it will be at least 3 years before the plant line 34-4 containing the *ipt* gene flowers. The strong gus expression exhibited by shoots of all four *Campanula* plants transformed with the *uidA* gene under the CaMV 35S promoter and the high levels of zeatin in transgenic plants containing *ipt* under the CaMV 35S promoter indicates that the CaMV 35S promoter directs very high levels of expression in leaves of *Campanula*.

The 35S-*ipt* gene transformation rate with LBA4404 was about five times higher than that with the *uidA* gene (Table 2). Transgenic plants were not recovered when 50 explants were co-cultivated with *Agrobacterium* containing the *ipt* gene controlled by either the heat-inducible promoter (hs) from the *Drosophila melanogaster hsp 70* gene (Smigocki 1991) or the wound-inducible promoter (PI-II) from the proteinase inhibitor II gene (Smigocki et al. 1993) (data not presented). These results suggest that the transformation yield may have been correlated with cytokinin level. The high endogenous levels of zeatin and zeatin riboside caused by transformation with the *ipt* gene under the strong CaMV 35S promoter appear to have sufficiently increased the cytokinin-to-auxin ratio to induce shoot formation (Smigocki and Owens 1988; Estruch et al. 1991).

In conclusion, *Agrobacterium tumefaciens* LBA4404 and EHA105 were equally effective in producing transgenic plants, but LBA4404 was preferable because of the difficulties in eliminating EHA105 from regenerated shoots of *Campanula*. A combination of 100 mg/l cefotaxime with 400 mg/l vancomycin was used following co-cultivation with *Agrobacterium* because these antibiotics effectively eliminated LBA4404 while maintaining the highest rate of regeneration (79% of the leaf explants) as compared to other antibiotics and concentrations. It was critical to apply an antibiotic and to apply it

at a concentration that did not negatively affect regeneration because the maximum level of regeneration from leaf explants of *Campanula* was much lower (3 shoots per leaf explant from 100% of the explants) than that observed for dicots such as tobacco. This system was successfully applied to the development of transgenic *Campanula* plants containing either the *uidA* or *ipt* genes.

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References

- Akiyoshi DE, Klee H, Amasino RM, Nester EW, Gordon MP (1984) T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proc Natl Acad Sci USA* 81:5994–5998
- Barry GF, Rogers SG, Fraley RT, Brand L (1984) Identification of a cloned cytokinin biosynthetic gene. *Proc Natl Acad Sci USA* 81:4776–4780
- Brandt K (1992) Micropropagation of *Campanula isophylla* Morretti. *Plant Cell Tissue Organ Cult* 29:31–36
- Brandt K (1994) Variation among and within clones in formation of roots and shoots during micropropagation of *Campanula isophylla*. *Plant Cell Tissue Organ Cult* 39:63–68
- Crook HC (1951) *Campanulas*, their cultivation and classification. Theophrastus Sakonnet, London
- DeBont A, Eggermont K, Druart P, DeVil M, Goderis I, van der Lerden J, Broekaert WF (1994) *Agrobacterium*-mediated transformation of apple (*Malus × domestica*): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant Cell Rep* 13:578–593
- Estruch JJ, Prinsen E, VanOnckelen H, Schell J, Spena A (1991) Viviparous leaves produced by somatic activation of an inactive cytokinin-synthesizing gene. *Science* 254:1364–1367
- Floriculture crops (1999) National Agricultural Statistics Service, Agricultural Statistics Board, U.S. Department of Agriculture. <http://usda.manlib.cornell.edu/report/nassr/other/zfc-bb/>
- Gan S, Amasino RM (1995) Inhibition of leaf senescence by auto-regulated production of cytokinin. *Science* 270:1986–1988
- Hoekema A, Hirsh PR, Hooykaas PJJ, Schilperoot RA (1983) A binary plant vector strategy based on separation of the vir- and T-regions of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303:179–180
- Hood EE, Helmer GC, Fraley RT, Chilton MD (1986) The hypovirulence of *Agrobacterium tumefaciens* A281 is encoded in the region of pTiBo542 outside the T-DNA. *J Bacteriol* 168:1291–1301
- Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* vector for plant transformation. *Transgenic Res* 2:208–218
- Ishimaru K, Ando M, Takamiya M, Terahara N, Yamakawa T, Shimomura K, Tanaka N (2001) Transgenic *Campanula* spp. (Bellflower). In: Bajaj YPS (ed) *Transgenic crops 3*. Biotechnology in agriculture and forestry, vol 48. Springer, Berlin Heidelberg New York, pp 55–68
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS-fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6:3901–3907
- Keel H, Horsch R, Rogers S (1987) *Agrobacterium*-mediated plant transformation and its further applications to plant biology. *Annu Rev Plant Physiol* 38:467–486
- Lewis P, Lynch M (1989) *Campanulas*. Helm, London
- Lulsdorf MM, Rempel H, Jackson JA, Balisk DS, Hobbes SLA (1991) Optimizing the production of transformed pea (*Pisum sativum* L.) callus using disarmed *Agrobacterium tumefaciens* strains. *Plant Cell Rep* 9:864–868

- Maheswaran G, Welander M, Hutchinson JF, Graham MW, Richards D (1992) Transformation of apple rootstock M26 with *Agrobacterium tumefaciens*. J. Plant Physiol 139:560–568
- Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning; a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Medford JI, Horgan R, El-Sawi Z, Klee HJ (1989) Alterations in endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. Plant Cell 1:403–413
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant 15:473–497
- Smigocki AC (1991) Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene. Plant Mol Biol 16:105–115
- Smigocki AC, Owens LD (1988) Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. Proc Natl Acad Sci USA 85: 5131–5135
- Smigocki AC, Neal JW, McCanna I, Douglass L (1993) Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene. Plant Mol Biol 23:325–335
- Tada H, Nakashima T, Kunitake H, Mori K, Tanaka M, Ishimaru K (1996) Polyacetylenes in hairy root cultures of *Campanula medium* L. J Plant Physiol 147:617–619
- Tanaka N, Matsuura E, Terahara N, Ishimaru K (1999) Secondary metabolites in transformed root cultures of *Campanula glomerata*. J Plant Physiol 155:251–254
- Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220:245–250
- Wang H, Qi M, Cutler AJ (1993) A simple method of preparing plant samples for PCR. Nucleic Acids Res 21:4153–4154